

# THE DISTRIBUTION OF RIFT VALLEY FEVER VIRUS IN THE MOSQUITO *CULEX PIPIENS* AS REVEALED BY VIRAL TITRATION OF DISSECTED ORGANS AND TISSUES

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**Abstract.** Distribution of Rift Valley fever virus (RVFV) was studied in the mosquito *Culex pipiens*. Mosquitoes were dissected on days 1-7 after an infectious bloodmeal, and RVFV plaque assays were performed on the legs, posterior midgut, ovaries, salivary glands, thoracic ganglia, and remaining organs and tissues (remnants). On days 7-12 and 14 following an infectious bloodmeal, mosquitoes were tested for their ability to transmit virus and then dissected. Dissemination (systemic infection) rates averaged 22% on days 1-14 and transmission rates 33% on days 7-14. There were no significant differences in the viral titers of midgut samples among the nondisseminated infected (virus limited to alimentary canal), disseminated infected nontransmitting, and transmitting groups of mosquitoes. The sequence of infection of the organs and tissues studied appeared to be as follows: midgut, hemolymph, remnants; salivary glands, ovaries, and thoracic ganglia. Some individuals were found to have disseminated infections as early as 12 hr following an infectious bloodmeal. Trauma, simulated by vigorous shaking immediately following the viremic bloodmeal, did not affect either infection or dissemination rates.

Rift Valley fever virus (RVFV) poses a major threat to livestock and humans throughout Africa. This virus is believed to be enzootic throughout much of the sub-Saharan region and is probably maintained by ground-pool *Aedes* spp.<sup>1</sup> During the 1977-79 Egyptian epidemic, however, *Culex pipiens* was implicated as the primary vector of RVFV.<sup>2,3</sup> Recent interest has, therefore, focused on this mosquito species.<sup>4</sup>

In general, virus first multiplies in the posterior midgut and is then released into the hemocoel where it infects other organs and tissues.<sup>5</sup> In several cases, dissemination of virus has occurred unexpectedly soon after a bloodmeal ("early" dissemination).<sup>5</sup> Recently, Eastern equine encephalitis (EEE) virions were seen in *Culiseta melanura* salivary glands by electron microscopy at 55-69 hr after an infective bloodmeal.<sup>6</sup> Also, Venezuelan equine encephalomyelitis (VEE) virions have been found in the fat body and epi-

dermis of *Cx. taeniopus* within 1 hr of engorgement.<sup>7</sup>

The objectives of this study were to determine the temporal and spatial distribution patterns of RVFV in transmitting and nontransmitting *Cx. pipiens* and to test for "early" dissemination.

## MATERIALS AND METHODS

### *Mosquitoes, virus, and assay procedures*

*Cx. pipiens* females (5-8 days old) (EL GABAL strain)<sup>17</sup> in the F<sub>48</sub> to F<sub>64</sub> generations were used in these experiments. Mosquitoes were reared and maintained in an incubator at 26 (±1)°C with a 16 hr photophase. Larvae were fed a mixture of yeast, liver powder, and ground hog chow. Adults were held in 3.8 liter cardboard containers, and cotton pledgets soaked in 10% sucrose provided a carbohydrate source. Moist gauze pads were placed on the screen lids, and containers were kept inside plastic bags to maintain high humidity.

Mosquitoes were infected by allowing them to feed on an anesthetized viremic hamster for approximately 1 hr. The hamster was inoculated intraperitoneally with ≈ 10<sup>4</sup> plaque forming units

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TABLE I  
Infection, dissemination, and transmission rates of *Cx. pipiens* orally exposed to RVFV

Day	Infection (No. infected/No. fed)			Dissemination (No. with virus in legs/No. infected)			Transmission	
	Dissected*	Not dissected†	%	Dissected	Not dissected	%	Rate % (No. transmitting/No. tested)	Transmission dissemination % (No. transmitting/No. with dissem. infection)
1	30/30	38/38	100.0	2/30	1/38	4.4	—	—
2	19/20	20/20	97.5	7/19	3/20	25.6	—	—
3	17/20	21/22	90.5	4/17	3/21	18.4	—	—
4	29/30	36/38	95.6	1/29	4/36	7.7	—	—
5	9/10	9/10	90.0	3/9	4/9	38.9	—	—
6	9/10	5/5	93.3	3/9	1/5	28.6	—	—
7	20/21	15/15	97.2	4/20	4/15	22.9	4/11 36.4‡	4/4 100
8	24/25	—	96.0	10/24	—	41.7	8/25 32.0	8/10 80
9	10/11	—	90.9	6/10	—	60.0	5/11 45.5	5/6 83.3
10	24/25	—	96.0	10/24	—	41.7	10/25 40.0	10/10 100
11	27/27	—	100.0	7/27	—	25.9	7/27 25.9	7/7 100
12	15/16	—	93.8	3/15	—	20.0	3/16 18.8	3/3 100
13	—	—	—	—	—	—	—	—
14	3/3	—	100.0	2/3	—	66.7	2/3 66.7	2/2 100

\* Mosquitoes dissected in 3 experiments.

† Mosquitoes in which only bodies and legs were assayed for virus in 3 experiments.

‡ Only 11 of 21 mosquitoes were tested for transmission.

(PFU) of RVFV about 24 hr prior to mosquito exposure.<sup>8</sup> The ZH 501<sup>9</sup> strain of RVFV had been passed twice in fetal rhesus lung cells before use in these experiments. Mosquitoes and mosquito parts for viral assay were triturated in 1 ml of mosquito diluent (10% calf serum in Medium 199 with Hanks' salt and antibiotics) and tested for infectious virus by plaque assay on 2–4-day-old Vero cell monolayers.<sup>8</sup> The mean amount of virus ingested by a sample of mosquitoes taken immediately following each infectious bloodmeal was taken as the viral "dose" for that experiment.

#### Dissection of mosquitoes

Mosquitoes were cold anesthetized, their wings and legs removed, and 0.2  $\mu$ l of hemolymph extracted by capillary action from the thorax and/or as a droplet at the base of a coxa. This sample was placed in 1.0 ml of diluent. Legs, posterior midgut, ovaries, salivary glands, thoracic ganglia, and remnants (remaining tissues) were dissected, washed 3 times in mosquito diluent, and then triturated in 1.0 ml of diluent for viral assay.

#### Dissemination and transmission experiments

The presence of virus in the legs and/or hemolymph sample was taken as an indication that

virus had disseminated from the midgut into the hemocoel.

For each of 3 experiments, between 500 and 1,000 mosquitoes were offered a bloodmeal on a viremic hamster (day 0), and 7 to 10 freshly engorged females were triturated for plaque assay to determine the mean amount of virus ingested ( $10^{4.4}$ ,  $10^{5.2}$ , and  $10^{5.7}$  PFU in experiments 1, 2, and 3, respectively). Mosquitoes ( $n = 10$ –30) were dissected on days 1–7. In one experiment, mosquitoes were tested for their ability to transmit virus on day 9. Subsequently on days 10–12 and 14, mosquitoes ( $n = 3$ –15) that fully engorged were dissected. In the other two experiments, engorged mosquitoes ( $n = 5$ –12) were dissected immediately following transmission tests on days 7–12. To supplement data collected from the dissected mosquitoes, legs and bodies of an additional sample were triturated on days 1–7 post-infectious bloodmeal (Table 1, "Not dissected").

#### Early dissemination experiments

To test for early dissemination, 400 mosquitoes were fed on a viremic hamster. Five freshly engorged mosquitoes were triturated to determine the mean amount of virus ingested ( $10^{6.7}$  PFU). At 12, 24, and 48 hr post-infectious bloodmeal, the legs from 100 females were removed

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TABLE 2

*Titer of RVFV recovered from selected tissues and organs of Cx. pipiens following transmission test with hamsters on days 7-14 following an infective bloodmeal (Exps. 1-3)\**

Tissue	Infected nontransmitters		Transmitters n = 34-39
	Nondisseminated n = 66-70	Disseminated n = 3	
Leg	0 (0)†	2.6 (2.3-3.0)	4.0 (2.8-4.8)
Midgut	3.2 (1.5-4.2)	3.3 (2.9-3.8)	3.2 (1.5-4.7)
Hemolymph	0	0.9 (0-1.3)	2.5 (0-3.4)
Salivary glands	0	0	4.0 (0-5.4)
Thoracic ganglion	0	0	2.7 (0-4.3)
Ovaries	0	0.3 (0-1.0)	2.1 (0-4.3)
Remnants	1.3 (0-3.7)	3.0 (2.5-3.5)	4.8 (2.9-5.5)

\* Mosquitoes ingested a mean of  $10^{4.4}$ ,  $10^{3.7}$ , and  $10^{3.1}$  in experiments 1, 2, and 3, respectively.

† Mean (range) of log<sub>10</sub> PFU/sample: 0 =  $10^{0.0}$  PFU/ml.

and titrated to determine if virus had disseminated from the midgut into the hemocoel.

To test the hypothesis that early dissemination may result from trauma as a result of laboratory handling, mosquitoes which had ingested an average of  $10^{4.5}$  PFU of virus from a hamster were separated into 3 groups of 30 individuals each. In group 1, the legs were assayed for virus 24 hr after feeding. Groups 2 and 3 were placed in separate 0.9 liter containers. Group 2 was agitated by vigorously shaking the container for 15 sec immediately following the bloodmeal; group 3 mosquitoes were not agitated. On day 3, the legs and bodies of mosquitoes in groups 2 and 3 were assayed for virus.

#### Data analysis

Infection, transmission, and dissemination rates were compared among experiments by day using Fisher's exact test.<sup>10</sup> Virus titers were compared among experiments by tissue by day using analysis of variance.<sup>10</sup> All tests were interpreted at the 0.05 significance level.

### RESULTS

#### *Distribution of virus and viral antigens in orally infected mosquitoes*

The results of the dissemination-transmission experiments were compared by day with regard to infection, dissemination, and transmission rates. No statistically significant differences were found, with the exception of the day 7 dissemination rate in experiment 1 vs. experiment 2.

Therefore, pooling the results of the 3 experiments (Table 1) was justified.

Overall, 96% (380/393) of the mosquitoes that took a viremic bloodmeal became infected. Dissemination rates (Table 1) ranged from 4.4% (3/68) on day 1 to 66.7% (2/3) on day 14. The overall dissemination rate was 21.6% (82/380) and the mean daily dissemination rate was  $31.0 \pm 18.4\%$ . Transmission rates (Table 1) varied from 18.7% (3/16) on day 12 to 66.7% (2/3) on day 14. The overall transmission rate was 33% (39/118). Among the 42 mosquitoes with disseminated infections tested, 39 (92.9%) transmitted virus.

The amount of virus detected in the various tissues from mosquitoes previously tested for their ability to transmit is shown in Table 2. The viral titers in each of the dissected tissues in the transmitter group were compared among experiments by day, and no statistically significant differences were found. In 3 of the transmitting mosquitoes, however, the viral titer of either the diluted hemolymph sample, the salivary glands, or the thoracic ganglia was  $<10^{1.0}$  PFU, which was not consistent with the viral titers of other tissues in these individuals. These tissues were probably lost during washing or transfer of the specimens to the tissue grinders.

There were no significant differences in infection, dissemination, and transmission rates by day among the 3 experiments. Further, we found no significant differences in viral titers among the 3 experiments by tissue by day in mosquitoes with nondisseminated infections, nontransmitting mosquitoes with disseminated infections, and mosquitoes that transmitted virus. Therefore the viral titers of the dissected tissues have been

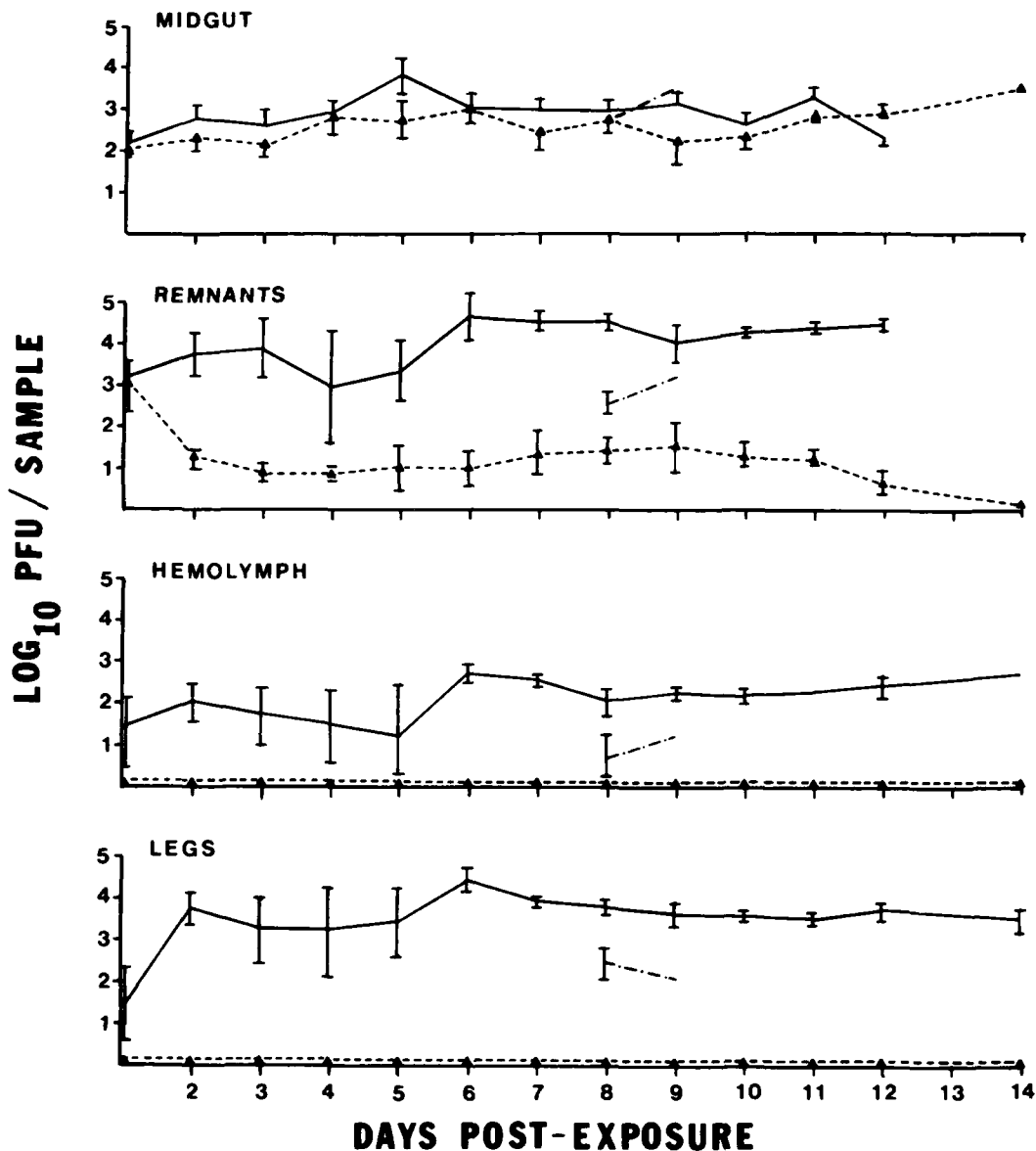


FIGURE 1. Mean titer ( $\pm$ SE) of RVFV in tissues dissected from *Cx. pipiens* on days 1-14 after feeding on viremic hamster. —, mosquitoes with disseminated infections; -- $\Delta$ -- $\Delta$ --, mosquitoes with nondisseminated infections (i.e., virus limited to alimentary canal); ---, mosquitoes with disseminated infections that failed to transmit virus.

combined for each day within each group and are presented graphically in Figures 1 and 2. Only the mosquitoes that became infected are represented on the graphs.

Among mosquitoes with nondisseminated infections, virus was recovered only from the midgut and remnants (Fig. 1). Five mosquitoes with

disseminated infections did not transmit virus. In 3 of these mosquitoes no virus was isolated from the salivary glands or the thoracic ganglia (Table 2). Virus was isolated from the salivary glands of the remaining 2 mosquitoes on days 10 and 14, but they did not transmit when fed on day 9. It was impossible to determine if they

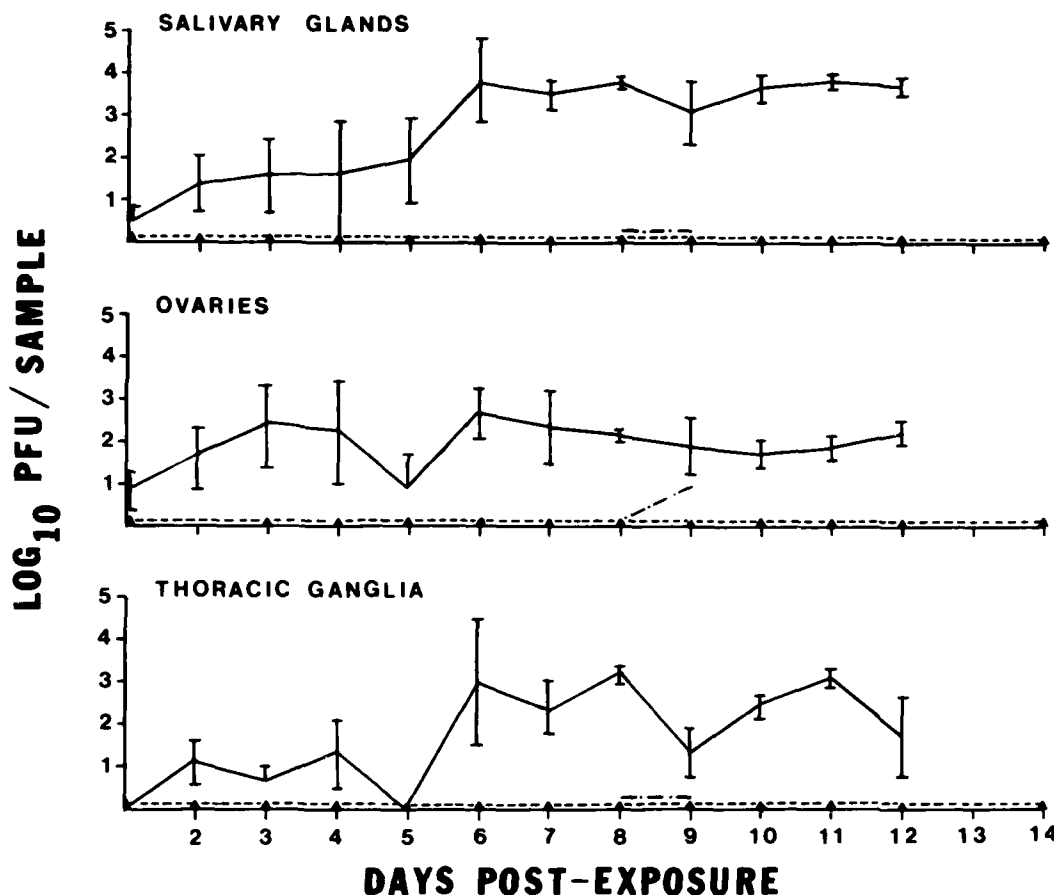


FIGURE 2. See Fig. 1 caption.

developed disseminated infections before or after the transmission test. These 2 mosquitoes are not included in Table 2 and were excluded from statistical analysis.

Viral titers of the midguts of infected mosquitoes ranged from  $10^{1.5}$ – $10^{4.7}$  PFU (Table 2).

In the majority of mosquitoes dissected, a small amount of blood was observed in the foregut diverticula immediately after bloodfeeding.

By 48 hr post-viremic bloodmeal, the mean viral titer in the remnants of individuals with disseminated infections was markedly higher than in those with nondisseminated infections (Fig. 1). In addition, mean viral titer in the remnants of transmitters was much higher than in both nontransmitters with disseminated infections and individuals with nondisseminated infections (Fig. 1).

Viral titration of dissected legs<sup>4</sup> and of he-

molymph were used to check dissemination status. Hemolymph samples from mosquitoes with disseminated infections contained from  $<10^{1.0}$ – $10^{3.4}$  PFU of virus (Table 2). When virus was detected in the hemolymph, virus was also present in the legs. However, there were specimens in which virus was present in the legs but not in the hemolymph samples.

Viral titers in the salivary glands and thoracic ganglia in transmitting specimens ranged from  $<10^{1.0}$ – $10^{5.4}$  PFU and from  $<10^{1.0}$ – $10^{4.3}$  PFU, respectively (Table 2). On 6 of the 14 days of the experiments, there was at least 1 individual with a disseminated infection that did not have detectable virus in the salivary glands or in the thoracic ganglia.

Virus recovered from the ovaries ranged from  $<10^{1.0}$ – $10^{4.3}$  PFU in transmitting individuals (Table 2). Other than an apparent increase from

TABLE 3

Early dissemination of RVFV in *Cx. pipiens* after ingesting  $10^{6.7}$  PFU of virus in a bloodmeal

Hours post-oral exposure	No. with virus in legs	Mean log <sub>10</sub> PFU RVFV in legs ± SD (range)
	No. tested (%)	
12	6/100 (6)	2.5 ± 0.7 (1.5–3.3)
24	9/100 (9)	3.0 ± 0.6 (2.0–4.0)
48	22/100 (22)	3.0 ± 1.0 (1.4–5.0)

day 1 to day 3, there was no consistent trend in the amount of virus associated with the ovaries over time (Fig. 2).

#### Early dissemination of RVFV

In one of the experiments carried out prior to those designed specifically to test for early dissemination, 1/20 mosquitoes had a disseminated infection 2 days after the viremic bloodmeal. In the individual with a disseminated infection, virus was found in all tissues examined:  $10^{2.9}$  PFU in hemolymph sample,  $10^{4.6}$  PFU in legs,  $10^{3.6}$  PFU in midgut,  $10^{3.3}$  PFU in ovaries,  $10^{3.0}$  PFU in salivary glands, and  $10^{4.6}$  PFU in the thoracic ganglia. The presence of virus in the salivary glands suggests that transmission might have been possible. Another individual had a disseminated infection as early as 24 hr post-viremic bloodmeal, but no virus was isolated from the salivary glands.

In an experiment designed specifically to test for early dissemination 6% (6/100) of the mosquitoes had disseminated infections as early as 12 hr post-viremic bloodmeal (Table 3). By 24 hr, 9% (9/100) and by 48 hr, 22% (22/100) had disseminated infections.

Rough handling (shaking of cage) did not have a significant effect on early dissemination (Table 4). All the females in the shaken cage survived, whereas 1 individual died in the unshaken, control cage. At 72 hr post-viremic bloodmeal there were somewhat more specimens with disseminated infections in the shaken than in the control cages; however, the difference was not statistically significant. In addition, the dissemination rate in the 72 hr agitated group was not significantly different from the 24 hr unagitated group.

In all tests for early dissemination, no blood was observed outside the alimentary canal, i.e., associated with dissected legs or visible through thin areas of cuticle.

TABLE 4

Effect of trauma by agitation on early dissemination of RVFV in *Cx. pipiens* after ingestion of  $10^{6.7}$  PFU of virus in bloodmeal

Hours post-oral exposure	No. with virus in legs	Mean log <sub>10</sub> PFU virus in legs ± SD (range)
	No. tested (%)	
24 (unagitated)	5/30 (17%)	3.1 ± 0.9 (2.0–3.8)
72 (unagitated)	2/24 (8%)	3.6 ± 1.6 (2.4, 4.7)
72 (agitated)	6/30 (20%)	4.2 ± 0.2 (4.0–4.4)

#### DISCUSSION

The graphs in Figures 1 and 2 suggest the following sequence of infection of the various organs and tissues studied: midgut, hemolymph, remnants; salivary glands, ovaries, thoracic ganglia. These results must be viewed somewhat cautiously since contamination with virus from the hemolymph is always a possibility. However, the occurrence of several specimens with disseminated infections, but with the salivary glands and thoracic ganglia uninfected, also supports this sequence.

In general, the pattern of dissemination we observed is consistent with the patterns seen in other mosquito/virus systems.<sup>5, 11–14</sup> Viremic blood is ingested and travels via the foregut through the proventriculus into the midgut. Virions then disseminate via the midgut epithelium into the hemolymph from which other tissues and organs, including the salivary glands, become infected.

Most mosquitoes examined had a small amount of blood in the foregut diverticula. Others have reported blood in the diverticula in different species.<sup>15–17</sup> Virus is unlikely to "escape" from the alimentary canal via the foregut because the foregut (including the diverticula) is lined with a chitinous intima that is impermeable even to water.<sup>18</sup> However, cells at the junction between the anterior midgut and the intussuscepted foregut, may, in addition to the midgut, act as conduits for virus into the hemocoel.<sup>19</sup> Infectious blood in the diverticula could be a source of infection in this region.

We found virus outside of the alimentary canal as early as 12 hr following an infectious bloodmeal in 6% of the sample tested. In a more recent study we have found virus in the legs of 6/50 (12%) of the mosquitoes tested as early as 4 hr after they ingested  $10^{6.2}$  PFU of virus (W. S. Ro-

moser, M. E. Faran, and C. L. Bailey, personal communication). The experiment in which a cage was vigorously shaken did not reveal an increase in dissemination rate, suggesting that early dissemination is not due to rough handling. However, this experiment did provide further evidence of early dissemination. We believe this is the first report of a bunyavirus disseminating in an insect host after such a short incubation period. Such early dissemination has, however, been reported to occur in other mosquito/virus systems.<sup>5</sup> In a recent study Weaver<sup>7</sup> observed, by electron microscopy, VEE virus (Togaviridae) in abdominal fat body adjacent to the posterior midgut within 1 hr after feeding, but no virus was seen associated with the basal lamina of the midgut until 3–4 hr post-engorgement. The presence of virions in the basal lamina would be consistent with prior release from midgut epithelial cells. In all early dissemination cases, virus appears to have entered the hemocoel prior to replication in the midgut epithelium. The mechanism of entry is unknown.

The variability in dissemination rate over time is consistent with the hypothesis that dissemination of RVFV from the midgut occurs sporadically after infection.<sup>20</sup> Once RVFV enters the hemocoel, it disperses and appears to replicate very rapidly, as indicated by the high titers which develop in mosquitoes that show early dissemination of virus (Tables 3, 4) and by previous work on *Cx. pipiens* intrathoracically inoculated with RVFV.<sup>4</sup> Such early dissemination is probably associated with early transmission. For example,  $10^{4.6}$  PFU of virus in the legs of an orally exposed mosquito incubated for approximately 48 hr at 26°C was well above the threshold ( $> 10^{4.2}$  PFU) we found necessary for this species to biologically transmit RVFV. It is also significant that this mosquito had  $10^{3.0}$  PFU of virus in its salivary glands. Virus associated with the salivary glands does not necessarily indicate the ability to transmit. However, virus was not found in the salivary glands of nontransmitting mosquitoes. Based on our results, and those of others,<sup>4</sup> the time between first appearance of RVFV in the hemocoel and infection of the salivary glands appears to be from <24 hr to 48 hr.

Since virus was detected more often in the legs than in both the legs and hemolymph samples, leg titration is apparently the more reliable indicator of dissemination status.

Virus was isolated from dissected ovaries, but we have not detected viral antigen in the follicular epithelia, oocytes, or nurse cells, but have detected antigen in cells of the lateral and common oviducts (W. S. Romoser, M. E. Faran, and C. L. Bailey, personal communication). It is also possible that the ovaries became contaminated with virus from the hemolymph.

In our study, 96% of the mosquitoes that took a viremic bloodmeal became infected. An earlier study revealed that 67% to 95% of the mosquitoes ingesting similar amounts of virus in earlier generations of this colony became infected.<sup>8</sup> The mosquitoes that failed to become infected may have been displaying a midgut infection (MI) barrier.<sup>11</sup> Of the infected mosquitoes, 21.6% developed disseminated infections and 92.9% of these transmitted virus to hamsters. The 78% that did not develop disseminated infections could be interpreted as indicating a so-called "midgut escape" (ME) barrier,<sup>11</sup> in that virus was limited to the alimentary canal and did not escape into the hemolymph. However, as mentioned earlier, specimens of *Cx. pipiens* appear to develop disseminated infections at varying times after virus exposure and, therefore, mosquitoes counted as having nondisseminated infections at one time may develop disseminated infections at a later time. Further, mosquitoes which failed to transmit virus may not have had disseminated infections long enough for salivary gland infections to develop. For example, *Cx. pipiens* with disseminated infections that did not transmit on days 7 or 8 post-viremic bloodmeal transmitted virus a week later.<sup>20</sup>

We would expect many of our results, e.g., overall pattern of dissemination and possibly the occurrence of early dissemination, to apply to field populations in a general way. However, in view of the changes observed in the same laboratory colony of *Cx. pipiens* in the  $F_1$  through  $F_{16}$  generations,<sup>8</sup> the quantitative results may not be representative of field populations.

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